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Humoral Immunity Patterns Based on Antibody Reactivity to Rotavirus Antigens in Brazilian Children Under 5 Years of Age

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The age distribution of antibody to simian rotavirus (SA-11) was studied in serum specimens obtained from 399 children aged to 5 years and living in the city of Recife (PE), located in the north eastern region of Brazil. Sera were examined for group-specific rotavirus antibody using a blocking enzyme immunoassay (bELISA) and a hemagglutination inhibition antibody (HIA) test, and for anti-VP2, anti-VP4, anti-VP6, and anti-VP7 antibodies using an immunoblotting assay (IBA). Antibody prevalence was similar in all bELISA and HIA assays, showing a steep rise in the 6- to 17-month-old age groups. The results indicate early acquisition of antibody to rotavirus. The majority of children aged 2 to 4 years had bELISA (50% to 60%) and HIA (70% to 81%) antibodies. There was an association in prevalence data obtained by HIA and bELISA with immunoblotting (IBA), revealing four serologic profiles. Children with profiles I and II (60%) respectively had HAI and ELISA antibody or HAI antibody alone and all had immunoprotective antibodies to VP4 and/or VP7. These children were regarded as "immune," resembling convalescent patients with a rotavirus infection. Children with profile III (4%) had no HIA antibody and only non-protective anti-VP6 and/or VP7 antibody, and were considered to be "partially immune." Children with profile IV (36%) had no detectable antibody and were classified as "nonimmune." These children should be considered to be susceptible to rotavirus infection, with the risk of developing clinically severe diarrhea.

KEY WORDS: rotavirus seroepidemiology, infants and young children, north eastern Brazil, gastroenteritis, immunoblotting of structural proteins, humoral immunity

INTRODUCTION

Group A rotaviruses are the most commonly recognized cause of severe diarrhea and death in infants and young children worldwide, particularly in developing countries [Kapikian and Chanock, 1990]. In Brazil, infant mortality is highest in the north eastern region, where it reaches 14%. More than 50% of all such recorded deaths are due to diarrhea as the major or associated cause of death [Puffer and Serrano, 1973; Yunes, 1981].

Live oral vaccines against rotaviruses are being developed with the goal of replicating natural infection and inducing immunity against diarrhea [Ward et al., 1990]. An important prerequisite for vaccine development is a better understanding of the mechanisms responsible for immunity, and of the sero-epidemiology of rotavirus infection in communities. Recently, O'Ryan et al. [1994] showed that specific serum IgA, IgG, and homotypic antibodies to rotavirus are correlated with protection against infection and illness, and Clemens et al. [1992] reported that serum IgG rotavirus antibodies induced by earlier infection were inversely related to the risk of clinically significant rotavirus diarrhea.

Most sero-epidemiologic studies on rotavirus infections have been conducted in developed countries [Kapikian and Chanock, 1990]. In Brazil, few such studies had been carried out and the immune response to these infections has not been completely characterized [Gomes et al., 1983; Ishak et al., 1984; Azeredo et al., 1989]. The aims of the present study were to determine the age distribution of rotavirus antibody in newborn to 5-year-old children living in the city of Recife (PE), north eastern region of Brazil, using hemagglutination inhibition and the blocking enzyme immunosorbent assays, and to establish the relationship between the antibody responses and the assays used.

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MATERIALS AND METHODS

Study Group and Serum Specimens

A blood sample was collected from 399 randomly selected newborn to 5-year-old children attending the General Pediatric Hospital of the Instituto Materno-Infantil de Pernambuco (IMIP), the main pediatric clinic in the city of Recife, for routine follow-up examination. All children were residents in one of 12 different districts of the metropolitan area of Recife. Specimens were collected by venepuncture during the months of September, October, and November of 1992. Children presenting acute or chronic gastrointestinal disease or a respiratory infection were excluded from the study. Six paired sera from patients (aged 6 months to 2 years) with a rotavirus infection were included in the study. All sera were stored at −20°C until transportation on dry ice to the Enteric Virus Laboratory of the Microbiology Department, Institute of Biomedical Sciences, University of São Paulo (ICB-USP). A panel of 10 sera with rotavirus antibody (as determined by bELISA and HIA), and 10 sera without rotavirus antibody were also included in order to provide additional positive and negative controls for the serologic assays.

Simian Rotavirus Antigen

Three batches of simian rotavirus (SA-11) antigen were produced by inoculating 0.01 m.o.i. trypsin-activated virus into confluent monolayers of the fetal monkey kidney (MA-104) cell line, containing Eagle's MEM with 1 μg/ml trypsin, as described previously [Hársi and Candeias, 1991]. Cells were harvested when a 90% cytopathic effect was evident and culture fluid was frozen and thawed twice. The culture fluid was then ultracentrifuged at 130,000*g* for 1 hr and 30 min. The pellets were dispersed with 0.1 M TRIS-HCl + 0.15 M $CaCl_2$ buffered saline (pH 7.3) and treated with an equal volume of Freon TF (Du Pont Co., Wilmington, DE, USA). Concentrates were divided into three parts. One portion was used for the hemagglutination inhibition assay (HIA) and no substance was added to the antigen. The second portion, used in the blocking enzyme immunoassay (bELISA), received an equal volume of 50% glycerol in phosphate buffered solution (pH = 7.4). The third portion for the immunoblotting assay (IBA) incorporated the following enzyme inhibitors: phenylmethylsulfonyfluoride, N-p-fosyl-L-lysine chloromethyl ketone, n-tosyl-L-phenyl alanine, antipain, leupeptin, and aprotinin, all at a final concentration of 20 μg/ml. Non-infected MA-104 cells were processed similarly to assess nonspecific reactivity.

Hemagglutination Inhibition Assay

HIA was carried out in U-shaped microtiter plates, as described previously [Ruiz-Gomes et al., 1981]. Heat-inactivated (56°C for 30 min) sera were diluted 1:8 in phosphate-buffered saline (PBS) at pH 7.4, and 4 hemagglutinating units of the SA-11 antigen were added to each serum dilution. After incubation for 1 hr at 37°C in a moist chamber, a 1% suspension of human group 0 erythrocytes was added to each reaction and incubated at room temperature for 1 hr. Two positive and two negative control sera were included in all tests.

Blocking Enzyme Immunoassay

bELISA was carried out in polystyrene microtitration plates according to Linhares et al. [1989] with some modifications. Plates were coated with goat hyperimmune serum to SA-11, at 1;10,000 dilution in 0.2 M carbonate-bicarbonate buffer, pH 9.6 (Bio-Manguinhos, Rio de Janeiro, Brazil) for 2 hr. After washing, serum specimens (diluted 1:20 in PBS with 0.5% tween, pH 7.4) were added to duplicate wells and an optimal dilution (1:64) of the SA-11 antigen was added to each well and incubated overnight at 4°C. The plates were washed on the following day and a 1:5,000 dilution of hyperimmune ginea-pig anti SA-11 serum was added and incubated at 37°C for 2 hr. After washing, a 1:6,000 dilution of anti-ginea-pig IgG, conjugated with peroxidase (Sigma Chemical Co., St. Louis, MO, USA) was added and incubated at 37°C for 2 hr. After a final wash, a chromogen substrate solution of o-pheniylene-diamine dihydrochloride and H_2O_2 was added and incubated for 10 min at room temperature. The reaction was stopped by adding 2 M H_2SO_4. Readings of optical density (OD) were performed with a Multiskan ELISA reader, using a 450 mm filter. The percentage of blocking value of each serum sample was determined as follows: percent blocking = (mean OD of 4 negative control sera - OD of sample)/(mean OD of 4 negative control sera - mean OD of 2 positive control sera) × 100. All samples that yielded a blocking value greater than 25% were considered to be positive.

Immunoblotting Assay

SA-11 virus antigen was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on 10% gel using a mini-slab gel system, according to Laemmli [1970]. Virus polypeptides were then transferred to a nitrocellulose membrane (NCM) sheet, according to the method described by Towbin et al. [1979]. The NCM sheets were cut into 3 mm strips and incubated overnight at 4°C with human sera diluted 1:100 in 10 mM Tris buffer (ph 7.5), as described by Ushijima et al. [1989]. One strip was incubated with a negative control serum and another was incubated with a positive control serum from a convalescent rotavirus diarrhea case. After washing three times in Tris buffer, NCM strips were incubated for 2 hr with a peroxidase-conjugated sheep anti-human IgG (gamma chain) (Biolab SA, Brazil), diluted 1:1,000. Strips were developed with freshly prepared 4-chloro-1-naphtol chromogen substrate (Sigma Chemical Co., USA). Protein molecular weight markers of 94 kDa, 67 kDa, 43 kDa, 30 kDa, 20 kDa, and 14 kDa. (Pharmacia Diagnostic AB, Sweden) and a guinea pig IgG to SA-11 VP6 protein, as well as a positive control serum were included to identify different viral bands.

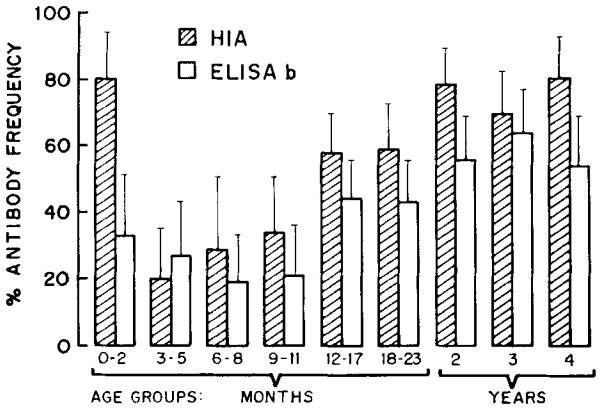

Fig. 1. Age distribution of human antibodies to simian rotavirus (SA-11) as measured by the hemagglutination inhibition assay (HIA) and by the blocking enzyme-linked immunosorbent assay (bELISA). The bars represent the 95% confidence limits. Recife (PE), Brazil, 1992.

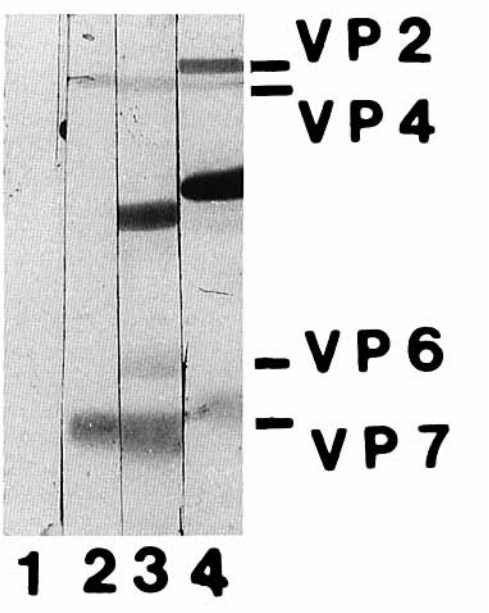

Fig. 2. Immunoblotting assay (IBA) of simian rotavirus (SA-11) against negative serum control **(lane 1),** positive serum sample from a child with rotavirus gastroenteritis recognizing viral peptides VP4 and VP7 **(lane 2),** positive serum samples from children recognizing viral peptides, VP4, VP6, and VP7 **(lane 3),** and VP2, VP4, and VP7 **(lane 4).**

Statistical Analysis

Reproducibility of the three batches of SA-11 antigen was checked against the panel of 10 positive and 10 negative rotavirus antibody sera. The kappa index (K) was determined and values higher than 0.9 were considered to be acceptable [Fleiss, 1981; Maclure and Willet, 1987]. Confidence intervals of antibody prevalences were calculated for the different age groups of the child population under study, at the 95% level [Feinstein, 1985]. Spearman's correlation coefficient was estimated for data obtained by HIA and bELISA [Rothman and Boice, 1989].

RESULTS

Age Distribution of Human Antibody

The prevalence of human antibody to SA-11 in different age groups was determined as shown in Figure 1. A total of 399 sera were tested for bELISA and HIA antibodies. The columns for each age group represent results obtained with 29 to 71 sera (30, 30, 31, and 29 sera from the 0- to 11-month-old age groups, respectively, and 71, 53, 61, 53, and 41 sera from the 1-to 4-year-old age groups, respectively). The similar patterns observed with the two serologicals tests are apparent (Fig. 1). Antibody present in the newborn to 2-month-old age group presumably was of maternal origin. The percentage of sera with rotavirus antibody decreased for the next several months, and was then followed by a sharp rise in the frequency of antibody. HIA antibody decreased at a precipitous rate, so that only 6 of 30 (20%) sera in the 3- to 5-month-old age group were positive. A decrease in bELISA antibody was also apparent, however, the decline was not as sharp as with HIA antibody, and thus the lowest level of ELISA antibody was noted in the 6 to 8-month-old age group.

Figure 1 also shows that bELISA and HIA antibodies were acquired commonly in the 6- to 17-month-old age groups. ELISA antibody was present in about 50% to 60% of children older than 23 months. HIA antibody was present in a high percentage of sera tested in the 2- to 4-year-old age groups (70% to 81%).

Immunoblotting in Sera From Healthy Children Without Gastroenteritis

Three batches of SA-11 antigen were produced in the present study and examined against a panel of 20 sera by bELISA and HIA, resulting in 100% agreement or K = 1.0 (z obtained = 3.65, z critical of 0.05 = 1.96) (data not shown). All three antigen preparations were also checked by IBA and presented reproducible antigenic bands of 86 kDa (VP4) and 34 kDa (VP7), when assayed against the positive sera. No band was seen when these antigens were assayed against negative sera (Fig. 2). Moreover, none of the three control antigens gave visible bands when tested against the same positive and negative sera.

Immunoblotting in Sera From Patients With Rotavirus Gastroenteritis

Six paired sera from children with confirmed rotavirus gastroenteritis were included in the present study as positive controls for the presence of VP2, VP4, VP6, and VP7 rotavirus IgG antibodies. At the onset of the disease (acute phase) VP4 and VP7 antibodies were found to be present in two of three positive sera by bELISA and HIA. Antibodies to VP4 and VP7 were also found in five of the six positive sera by bELISA and HIA in the convalescent phase of disease (2 to 3 weeks later). Rotavirus VP4 and VP7 antibodies, which confer protection, as well as VP2 and VP6 non-protective antibodies detected in children are illustrated in Figure 2.

TABLE I. Serologic Profiles in Response to Rotavirus Infection, According to An Association in Prevalence Data Obtained by HIA and bELISA With Immunoblotting (IBA), in Newborn to 5-Year-Old Children Living in Recife (PE), Brasil

Serologic profile	Presence of antibody: Number of positive sera	(%)	Presence of IgG to viral peptides	Humoral Immunity status
I) HIA and bELISA positive	159	(40)	VP4 and/or VP7, VP2, VP6	"immune"
II) Only HIA positive	80	(20)	VP4 and/or VP7	"immune"
III) Only bELISA positive	16	(4)	VP6 and/or VP7	"partially immune"
IV) HIA and bELISA negative	144	(36)	None	"Non-immune"
TOTAL	399	(100)		

Immunoblotting Assay (IBA) and Its Relationship to bELISA and HIA

The examination of 110 human sera selected at random for antibody responses to SA-11 rotavirus provided an opportunity to compare the prevalence of positive reactions by bELISA and HIA with those obtained by the immunoblotting assay (BIA). As shown in Table I, there was an association in prevalence data obtained by bELISA and HIA with immunoblotting, revealing four serologic profiles. In profile I, bELISA and HIA positive sera had antibodies that recognized all four viral antigenic bands (VP2, VP4, VP6, and/or VP7). In profile II, bELISA negative and HIA positive sera had antibodies that recognized only two antigenic bands (VP4 and/or VP7). In profile III, bELISA positive and HIA negative sera had antibodies that recognized only VP6 and/or VP7. In profile IV, bELISA and HIA negative sera had antibodies that presented no reactivity to any virus antigenic bands.

As shown in Figure 3, profiles I and II varied among the different age groups, showing patterns similar to those observed with bELISA and HIA. Profile III had an almost equal frequency of only 4% in the majority of age groups. Profile IV showed high frequencies (62 to 65%) in the 3- to 11-month-old age groups and decreased to 18% in the 4-year-old age group.

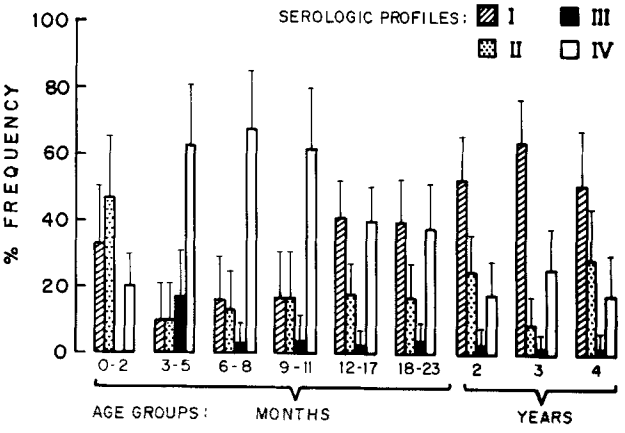

Fig. 3. Serologic profiles to rotavirus infection in children from different age-groups. Recife (PE), Brazil, 1992. Profile I: HIA and bELISA positive, profile II: HIA positive and bELISA negative, profile III: HIA negative and bELISA positive, and profile IV, HIA and bELISA negative. The bars represent the 95% confidence limits.

DISCUSSION

The present study indicates that antibody to rotavirus is acquired at an early age and supports the concept that a high infection rate occurs early in life. A majority of the 3- to 4-year-old children studied had antibodies to the virus as measured by two separate assays. However, it is not known whether the antibody present in these age groups is due to a prolonged antibody response or to reinfection.

The finding that bELISA and HIA antibodies were acquired commonly by children in the 6- to 17-month-old age groups is consistent with the observation made in north eastern Brazil that rotavirus infection rates are highest in the same age groups [Stewien et al., 1991; Magalhães et al., 1990]. In a prospective study, Linhares et al. [1989] defined four main patterns of acquisition of rotavirus bELISA antibody in children followed up from birth to the age of 3 years. First, children with "long-term" immunity (62%), second, children with "short-term" immunity (18%), third, children with no antibody response (12%), and fourth, children who were seropositive since birth (8%). They observed that children with "short-term" immunity can become susceptible again by loosing antibody and be reinfected once or twice with rotaviruses.

The results support and expand the findings of two other serological surveys of bELISA antibody and one of HIA antibody in serum specimens collected from infants and young children in three diffent cities in Brazil [Azeredo et al., 1989; Ishak et al., 1984; Gomes et al., 1983]. The antibody patterns seen in the city of Recife are almost similar to those observed in other developing and developed regions of the world. The frequency of antibody positive children was similar to that found in the United States, Germany, and Bangladesh [Blacklow et al., 1976; Brussow et al., 1988; Sack et al., 1980], somewhat higher than in Thailand [Echeverria et al., 1983], and lower than in Mexico [Ruiz-Gomes et al., 1981]. Although there was a significant association between the bELISA and HIA tests (77% agreement), con-

cordance between these assays was only moderate in terms of K index (0.5). It is evident that the use of bELISA will tend to miss more serological responses than will the use of HIA. Moreover, there were major differences in the antibody responses detected by these two assays in the present study. bELISA detects antibody to the common group A inner capsid peptide VP6 of the viral particle, while the HIA detects antibody to the outer capsid peptide VP4, which has hemagglutinating and major neutralizing activities, according to Ward et al. [1993]. Thus, bELISA only evaluates antibody to recent or past rotavirus infections, whereas HIA also appraises humoral immunity. It is concluded that the use of HIA in sero-epidemiological surveys gave a better and a more complete picture of antibody and immunity levels in the population under study.

The immunoblotting assay (IBA) was used to analyze the polypeptides of rotavirus recognized by serum antibodies detected in children. Rhesus rotavirus strain SA-11 was used because it is immunologically cross-reactive to human rotavirus [Kapikian and Chanock, 1990]. Immune responses to SA-11 structural polypeptides were studied for specific IgG, IgM, and IgA in paired sera from children with and without gastroenteritis [Ushijima et al., 1989]. These investigators showed that IBA is useful for detecting antibodies in human rotavirus infections at the viral peptide level, especially structural polypeptides VP2, VP4, VP6, and VP7.

In the present study we established four serologic profiles on the basis of the association of prevalence data obtained by bELISA and HIA with those obtained by immunoblotting (IBA). As shown in Table I, 40% of children were found to have antibodies by both tests, 20% exhibited antibody only by HIA, 4% had antibody only by bELISA, and 36% were negative by both tests. These serologic profiles identified three patterns which are consistent with the humoral immune status of the children examined (Table I).

Children with profile I or II had protective antibodies to VP4 and/or VP7 and thus were considered to be "immune," since their profile was similar to that of convalescent patients with gastroenteritis. Anti-VP4 antibodies were not found by IBA in one of six convalescent patients with gasteroenteritis, three of 36 children with profile I and 17/30 children with profile II, although they all had HIA antibodies. This can be explained by the lower sensitivity of IBA which detects immobilized antibody, and by the high dilution factor used in the tests (1:100). Children with profile III had only non-protective anti-VP6 and/or anti-VP7 antibody. They were considered "partially immune" because they lacked anti-VP4 antibody.

Finally, children with profile IV had no detectable antibody and therefore were classified as "nonimmune". As shown in Figure 3, the frequency of this pattern was high in children aged 3 to 11 months (>60%), and declined gradually in the next age-groups down to 18% in 4-year-old children, which is consistent with the concomitant increase of profiles I and II. It is concluded that there is still a significant fraction in this age-group without protective anti-VP4 and anti-VP7 antibodies, which is at risk of diarrheal disease.

The high prevalence of seropositivity (profiles I + II = 80%) in 0- to 2-month-old infants (Fig. 3) may explain at least in part why immunization against rotavirus has thus far not been very successful in this age group [Black, 1993; Migasena et al., 1995]. Our data also show that best possible immunization results would be expected when live oral rotavirus vaccine is first given to infants aged 3 to 5 months, when prevalence of seronegativity and "partial immunity" (profiles III + IV) are maximal (62 + 18 = 80%). In addition, the finding that a significant rate of children (18%) remained without antibodies in the 4-year age-group, reinforces the view that several exposures to rotaviruses, and consequently to the live oral rotavirus vaccine, are needed for the development of high levels of immunity and protection against the disease.

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